

Homodimerization Is Essential for the Receptor for Advanced Glycation End Products (RAGE)-mediated Signal Transduction*

Received for publication, April 14, 2010, and in revised form, May 24, 2010. Published, JBC Papers in Press, May 26, 2010, DOI 10.1074/jbc.M110.133827

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The receptor for advanced glycation end products (RAGE) is a pattern-recognition receptor that binds to diverse ligands and initiates a downstream proinflammatory signaling cascade. RAGE activation has been linked to diabetic complications, Alzheimer disease, infections, and cancers. RAGE is known to mediate cell signaling and downstream proinflammatory gene transcription activation, although the precise mechanism surrounding receptor-ligand interactions is still being elucidated. Recent fluorescence resonance energy transfer evidence indicates that RAGE may form oligomers on the cell surface and that this could be related to signal transduction. To investigate whether RAGE forms oligomers, protein-protein interaction assays were carried out. Here, we demonstrate the interaction between RAGE molecules via their N-terminal V domain, which is an important region involved in ligand recognition. By protein cross-linking using water-soluble and membrane-impermeable cross-linker bis(sulfosuccinimidyl) suberate and nondenaturing gels, we show that RAGE forms homodimers at the plasma membrane, a process potentiated by S100B and advanced glycation end products. Soluble RAGE, the RAGE inhibitor, is also capable of binding to RAGE, similar to V peptide, as shown by surface plasmon resonance. Incubation of cells with soluble RAGE or RAGE V domain peptide inhibits RAGE dimerization, subsequent phosphorylation of intracellular MAPK proteins, and activation of NF- κ B pathways. Thus, the data indicate that dimerization of RAGE represents an important component of RAGE-mediated cell signaling.

The receptor for advanced glycation end products (RAGE)² was first cloned as an AGE-binding protein (1), but it is now evident that this so-called pattern-recognition receptor also

binds to multiple ligands, including S100 proteins, amphoterin/high mobility group box-1, amyloid- β , and Mac-1 (2–5). RAGE is expressed in a wide range of tissues, such as brain, kidney, liver, heart, and the vasculature, and in diverse cell types, including smooth muscle cells, endothelium, T-lymphocytes, neurons, and monocytes/macrophages (6). The receptor may have a homeostatic role as a component of the innate immune response (7). Overexpression of RAGE and downstream proinflammatory signaling upon ligand binding are associated with several disease states such as diabetic complications, Alzheimer disease, cancer, and viral infections (4, 7–11). Soluble RAGE (sRAGE), which is composed of the extracellular domains but lacks the transmembrane and cytosolic domains, is produced by both proteolytic cleavage of RAGE and alternative mRNA splicing (12, 13). Soluble RAGE may act as a dominant negative isoform and block RAGE signaling by functioning as an extracellular “decoy receptor” to inhibit RAGE ligand binding (14).

The human RAGE gene (or AGER) is located on chromosome 6 in the major histocompatibility complex class III region that contains 11 exons. RAGE consists of 404 amino acids with a molecular mass of 45–55 kDa, which can be variable depending on differential glycosylation states (1). Structurally, RAGE belongs to the immunoglobulin superfamily, and the protein consists of an N-terminal signal peptide (amino acids 1–22), a V-type immunoglobulin-like domain (amino acids 23–116), two tandem C-type immunoglobulin-like domains (amino acids 124–221 and 227–317, respectively), a single transmembrane domain (amino acids 343–363), and a short C-terminal intracellular cytoplasmic tail (amino acids 364–404) (1). The V domain is the binding site for AGEs that interact with RAGE at the micromolar level (9), although other domains also play a role in ligand binding. For example, S100B binds to both the V and C1 domains, whereas S100A6 binds to C1 and C2 domains (15). It was reported recently that the V and C1 domains are not independent, but rather form an integrated structural unit for ligand recognition (16).

The cytoplasmic tail is essential for RAGE-mediated cell signaling, although this sequence has no homology to any known protein-tyrosine or serine/threonine kinase motif. Recent evidence using a yeast two-hybrid approach suggests that the RAGE cytoplasmic domain interacts with diaphanous-1 (Dia-1), and this potential binding partner could constitute the basis for intracellular signaling (17). RAGE-ligand binding results in phosphorylation of various protein kinases involving MAPKs, Rac/Cdc42, and Janus kinase (JAK)/signal transducers and acti-

* This work was supported by the Juvenile Diabetes Research Foundation and Fight for Sight (United Kingdom).

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² The abbreviations used are: RAGE, receptor for advanced glycation end products; AGE, advanced glycation end product; sRAGE, soluble RAGE; MAPK, mitogen-activated protein kinases; SPR, surface plasmon resonance; JNK, c-Jun N-terminal kinases; MEK, MAPK/ERK kinase; NF- κ B, nuclear factor κ B; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, the influenza hemagglutinin epitope; BS³, bis(sulfosuccinimidyl) suberate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DTT, dithiothreitol; RU, resonance unit.

vator of transcriptions (STATs) and subsequently activates the NF- κ B pathway, which can be attenuated by the extracellular domain of RAGE (18–23). Such signal transduction pathways link RAGE to a number of inflammation-related cell responses, such as apoptosis, mobility, migration, and proinflammatory gene expression (24). Thus, RAGE has been the focus for a number of small molecule drugs or neutralizing antibodies that can regulate ligand binding or downstream signal transduction and thereby prevent disease (24, 25).

Most RAGE ligands, including S100B and amyloid β , function as oligomers (26, 27). A recent study using in-cell fluorescence resonance energy transfer (FRET) has demonstrated that RAGE forms oligomers at the plasma membrane (28). This property may be necessary for AGE ligand recognition and signal transduction, albeit in a manner dissimilar from the well characterized phosphotyrosine kinase receptors for many growth factors and cytokines (29). It has been shown that bacterially expressed sRAGE form tetramers *in vitro* (30), but to date, very little experimental evidence has been provided to identify the oligomerization of RAGE on the cell surface. This study sought to determine the nature of RAGE oligomerization and whether this property is altered by ligand binding and required for downstream signaling. Our findings add to understanding the molecular nature of RAGE and highlight possibilities for novel intervention strategies.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatments, and Transfections—HEK293T cells from the American Type Culture Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS) and 100 μ g/ml PrimocinTM and were maintained in a humidified incubator containing 5% CO₂ at 37 °C. Approximately 1×10^6 HEK293T cells were plated in each well of a 6-well plate in 10% FCS/DMEM for 24 h. Cells were then cultured in 1% FCS/DMEM for another 24 h followed by S100B or AGE-BSA treatment.

For transfection of plasmids in HEK293T cells, 3×10^6 cells were plated on 100-mm Petri dishes at 24 h before transfection. DNA-Lipofectamine 2000 complex was prepared by mixing 1 ml of Opti-MEM I reduced serum medium containing HA-RAGE and GFP-RAGE plasmids (4 μ g each) with 1 ml containing 30 μ l of Lipofectamine 2000 (Invitrogen) and incubating the mixture at room temperature for 20 min. DNA-Lipofectamine 2000 complex was added to the wells containing cells and 10% FCS/DMEM. 24 h post-transfection, cells were harvested for immunoprecipitation or cultured for another 24 h in 1% FCS/DMEM followed by S100B or AGE-BSA for further analysis.

Plasmids—Full-length of RAGE cDNA was isolated from the human retinal Müller glial cell line (MIO-M1; a kind gift from Dr. Astrid Limb, Institute of Ophthalmology, University College London, UK) using primers 5'-AAGGAATTCATGGCAGCCGGAACAGCAGTTGGAGC-3' and 5'-ATTCTCGAGTCAAGGCCCTCCAGTACTACTCTC-3' and inserted into EcoRI/XhoI restriction sites of pcDNA3-GFP, pcDNA3-HA, pGEX-4T-1 vectors. RAGE deletions, which were termed as NM (amino acids 1–363, sense primer 5'-AAGGAATTCATGGCAGCCGGAACAGCAGTTGGAGC-3' and antisense

primer 5'-AGTCTCGAGTTACTTCCCAGGAATCTGGTAGACAC-3'), CM (amino acids 318–404, sense primer 5'-CTAGAATTCATCAGCATCATCGAACCAGGCG-3' and antisense primer 5'-ATTCTCGAGTCAAGGCCCTCCAGTACTACTCTC-3'), V (amino acids 1–123, sense primer 5'-AAGGAATTCATGGCAGCCGGAACAGCAGTTGGAGC-3' and antisense primer 5'-AGTCTCGAGTTACTTCCCAGGAATCTGGTAGACAC-3'), C (amino acids 124–342, sense primer 5'-GCCGAATTCAGAAATTGTAGATTCTGCCTC-3' and antisense primer 5'-ATTCTCGAGTTAGGCTAGATTCCCAGCCCTGATC-3'), C1 (amino acids 124–226, sense primer 5'-GCCGAATTCAGAAATTGTAGATTCTGCCTC-3' and antisense primer: 5'-ATTCTCGAGTTACTGGATGGGGGCTGTGCGCAAG-3'), C2 (amino acids 227–342, sense primer 5'-ATAGAATTCCTCGTGTCTGGAGCCTGT-3' and antisense primer 5'-ATTCTCGAGTTAGGCTAGATTCCCAGCCCTGATC-3'), were PCR-amplified, and subcloned into EcoRI/XhoI restriction sites of pcDNA3-GFP, pcDNA3-HA, and pGEX-4T-1 vectors. For RAGE cysteine mutants, cysteine at amino acids 38, 99, 144, 208, 259, or 301 was mutated to alanine individually by site-directed point mutagenesis and subcloned into pcDNA3-HA vector. The cis-reporter plasmid pNF κ B-Luc (Stratagene) encodes a *Photinus pyralis* (firefly) luciferase under a regular TATA box and an enhancer element with a synthetic promoter of five tandem NF- κ B-binding sites. The pRL plasmid (Promega) contains a *Renilla reniformis* (*Renilla*) luciferase cDNA under the control human cytomegalovirus promoter.

Immunofluorescence—HEK293T cells were plated onto coverslips in a 6-well plate. Cells were fixed in 4% paraformaldehyde for 1 h at room temperature followed by permeabilization using 0.2% Triton X-100, 5% BSA/PBS for 5 min on ice. The coverslips were then blocked in 5% BSA/PBS for 1 h at room temperature. After a brief washing, the coverslips were incubated with mouse monoclonal anti-RAGE antibody (1:1000) (Millipore) at 4 °C overnight, washed, and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:500) (Invitrogen) in a dark chamber for 1 h at room temperature. After extensive washing, the coverslips were inverted and mounted on slides with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories). The immunofluorescence was assessed using Nikon TE-2000 confocal microscopy.

Preparation of AGEs—AGE-modified albumin was prepared as described previously (31). 50 mg/ml BSA was incubated with 0.5 M of D-glucose in 0.2 M PBS, pH 7.4, at 37 °C for 60 days. Control albumin was incubated for a comparable time in PBS alone. AGEs were dialyzed in 0.2 M sodium phosphate buffer, pH 7.4, and were passed through a Detoxi-Gel endotoxin removing column (Pierce). The protein concentration was determined using BCA quantification kit (Pierce).

Western Blotting and Immunoprecipitation—HEK293T cells maintained in 1% FCS/DMEM were treated with the indicated amount of S100B or AGE-BSA for 30 min. Cell lysates were prepared with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 5 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM NaF, 1 \times protease inhibitor mixture) on ice for 30 min. 20 μ g of the total protein was applied to a 10% SDS-PAGE and blotted to an Immobilon-FL

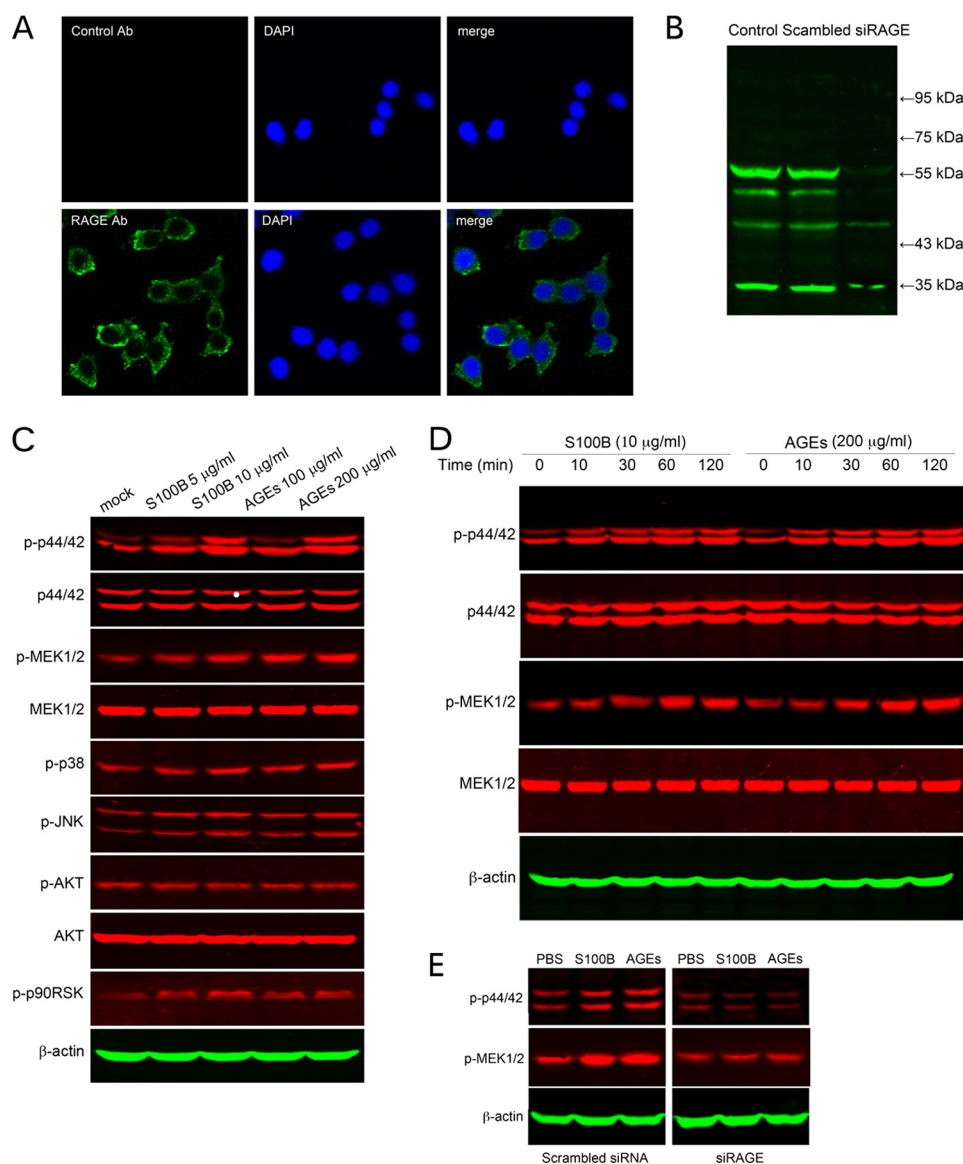


FIGURE 1. RAGE-activated MAPK pathways in HEK293T cells. A, 4% paraformaldehyde-fixed HEK293T cells were evaluated for RAGE immunolocalization using an anti-RAGE monoclonal antibody (Ab) (green). The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). S, signal peptide; V, IgV-like domain; C, IgC-like domain; M, transmembrane region; T, cytosolic tail. B, HEK293T cells were transfected with 20 nmol scrambled or RAGE-specific siRNA. 48 h post-transfection, cells were lysed and subjected to Western blotting for RAGE. C, HEK293T cells were cultured in 1% FCS/DMEM for 24 h followed by treatment with the indicated amounts of S100B or AGE-BSA for 30 min. Western blotting for total and phosphorylated p44/42, MEK1/2, p38, JNK, p90RSK, Akt, and β -actin was performed using 20 μ g of the whole cell lysates. D, HEK293T cells were cultured in 1% FCS/DMEM for 24 h followed by treatment with 10 μ g/ml S100B or 200 μ g/ml AGE-BSA for the indicated time. Phosphorylated or total p44/42 and MEK1/2 was probed using 20 μ g of the whole cell lysates. E, HEK293T cells were transfected with 20 nmol of scrambled or RAGE-specific siRNA. 24 h post-transfection, cells were grown in 1% FCS/DMEM for another 24 h and then treated with 10 μ g/ml S100B or 200 μ g/ml AGE-BSA for 30 min. Phosphorylation of p44/42 and MEK1/2 was detected using 20 μ g of the whole cell lysates.

polyvinylidene difluoride membrane (Millipore). Quantitative Western blotting was performed using Odyssey infrared imaging system (Li-COR), which allows detection of two proteins simultaneously by using fluorescently labeled antibodies and quantification of proteins more accurately over a much wider linear dynamic range than chemiluminescence by using near-infrared fluorescence detection (32). Briefly, the Immobilon-FL polyvinylidene difluoride membrane was incubated with rabbit monoclonal anti-p44/42 (1:1000) or p-p44/42 (1:1000) antibody (Cell Signaling Technology) together with mouse mono-

clonal β -actin antibody (1:20,000, Sigma) at 4 °C overnight followed by incubation with IRDye 680 goat anti-rabbit and IRDye 800CW goat anti-mouse secondary antibodies (1:10,000) (Li-COR) at room temperature for 30 min. The membrane was then scanned, and the bands were quantified using the Odyssey imaging system.

For immunoprecipitation, HEK293T cells were transfected with GFP-RAGE and HA-RAGE or pcDNA3-HA vector, treated with RAGE ligands as aforementioned, and lysed in RIPA buffer. 500 μ g of the total cell lysates were precleared with protein A/G Plus beads (Santa Cruz Biotechnology) for 1 h at 4 °C and then incubated with anti-HA antibody (Roche Applied Science) and protein A/G Plus beads overnight at 4 °C. The beads were extensively washed, eluted by boiling in SDS protein sample buffer, and subjected to SDS-PAGE and Western blotting.

Protein Purification, Cleavage, and GST Pulldown—pGEX-4T-1-RAGE, pGEX-4T-1-sRAGE, and pGEX-4T-1-V were transformed into BL21 *Escherichia coli*. Transformed *E. coli* cells were induced by 0.2 mM isopropyl 1-thio- β -D-galactopyranoside for 4 h at 37 °C. Cells were harvested and lysed in Buffer A (25 mM Tris-HCl, pH 8.0, 1 mg/ml lysozyme, 1 mg/ml DNase I, 100 mM NaCl, 1 mM DTT, 1 mM PMSF). The pellet was collected by centrifugation and washed with Buffer B (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM PMSF, 1% Triton X-100) and Buffer C (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM PMSF, 2 M urea). The pellet was then resuspended in Buffer D (50 mM Tris-HCl, pH 8.0,

10 mM DTT, 8 M urea). The aqueous fraction was dialyzed with gradient urea buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT supplemented with 6, 4, 3, 2, 1, 0 M of urea, respectively). The dialyzed samples were centrifuged, and the GST fusion proteins in the supernatants were purified with MagneGST beads (Promega) according to the manufacturer's instructions. Purified proteins were cleaved as required using Thrombin CleanCleave kit (Sigma) according to the manual. Cleaved GST tag was cleared from the protein samples by MagneGST beads.

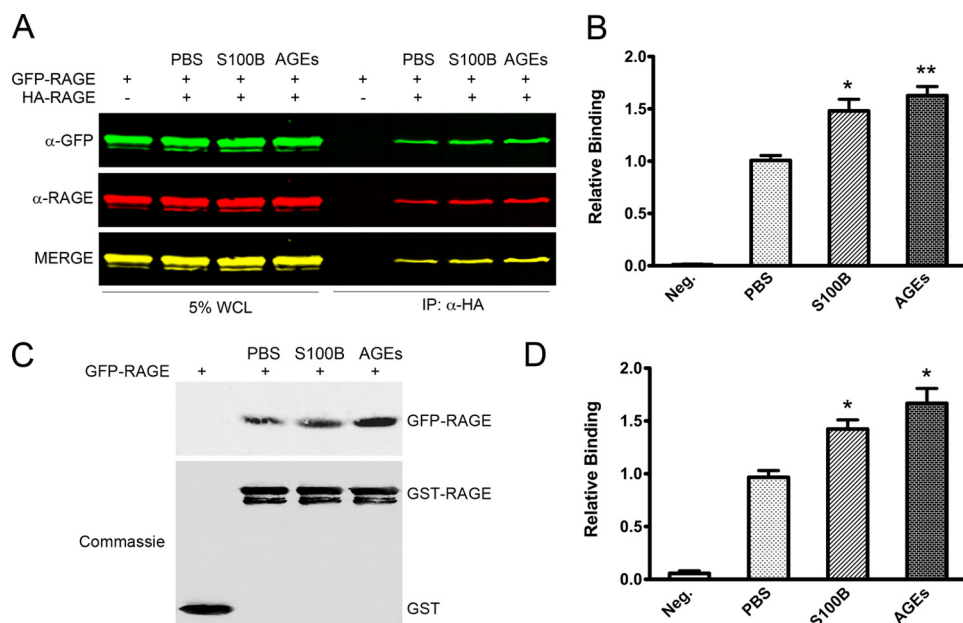


FIGURE 2. RAGE interacts with itself in cells and *in vitro*. *A*, HEK293T cells were co-transfected with GFP-RAGE and HA-RAGE or pcDNA3-HA vector. 24 h post-transfection, cells were grown in 1% FCS/DMEM and treated with 10 μ g/ml S100B or 200 μ g/ml AGE-BSA for 30 min. Cells were then lysed in RIPA buffer and subjected to immunoprecipitation (IP) using anti-HA antibody. The immunoprecipitates were probed with anti-GFP (green bands) and anti-RAGE antibodies (red bands). WCL, whole cell lysate. *B*, quantitation of the interaction between HA-RAGE and GFP-RAGE as shown in *A* by Odyssey infrared imaging system. *, $p < 0.05$; **, $p < 0.01$ for S100B or AGE-treated cells versus PBS-treated cells. Data are presented as mean \pm S.E. of triplicate experiments. *Neg.*, negative. *C*, GST-RAGE or GST control protein was expressed in *E. coli*, purified, immobilized onto glutathione-Sepharose 4B beads, and incubated with the cell lysates obtained from GFP-RAGE-transfected HEK293T cells. The proteins eluted from the beads were applied to Western blotting with anti-GFP antibody (upper panel). Immobilized GST and GST-RAGE were stained with Coomassie Brilliant Blue R-250 (lower panel). *D*, quantitation of the interaction between GFP-RAGE and purified GST-RAGE as shown in *C* by Odyssey imaging system. *, $p < 0.05$ for S100B or AGE-treated cells versus PBS-treated cells. Data are mean \pm S.E. of triplicate experiments.

HEK293T cells were transfected with a GFP-RAGE expression plasmid in 100-mm Petri dishes. 24 h after transfection, cell lysates were prepared with RIPA buffer and incubated with GST-fused RAGE, sRAGE, or V peptide, which had been pre-immobilized to glutathione-Sepharose 4B beads. The protein-bound beads were extensively washed and then eluted in SDS protein sample buffer at 95 °C for 5 min. The eluates were loaded onto an SDS-polyacrylamide gel.

Protein Cross-linking—Protein cross-linking using bis(sulfo-succinimidyl) suberate (BS³) was performed according to the manufacturer's manual. Briefly, HEK293T cells were harvested in PBS at a density of 2×10^7 cells/ml, washed three times with ice-cold PBS, and then incubated with 4 mM BS³ for 30 min at room temperature. The reaction was terminated by adding 20 mM Tris-HCl, pH 7.5, for 15 min at room temperature. Cells were then lysed in RIPA buffer, and Western blotting was performed by loading 20 μ g of the whole cell lysates on an 8% SDS-polyacrylamide gel.

Nondenaturing PAGE—Native gels containing 8% acrylamide were prepared in 0.375 M Tris-HCl, pH 8.8, without SDS. The cell lysates were mixed with 5 \times native gel sample buffer (312.5 mM Tris-HCl, pH 6.8, 0.05% bromophenol blue, 10% glycerol). The gel loaded with 20 μ g of the total protein was run in native PAGE buffer (25 mM Tris-HCl, 192 mM glycine). Proteins were then transferred to an Immobilon-FL polyvinylidene difluoride membrane. Western blotting was performed as described above.

Surface Plasmon Resonance—Protein binding interactions were performed using a surface plasmon resonance (SPR) Biacore Q system (Biacore) as described previously (15) with minor modifications. GST and GST-RAGE were immobilized onto distinct flow cells on the surface of CM5 chips (Biacore) via amine coupling with the carboxyl groups of the chip dextran surface activated with 1:1 (v/v) solution of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 50 mM *N*-hydroxysuccinimide for 7 min at a flow rate of 5 μ l/min. 75 μ l of protein to be immobilized (50 μ g/ml) in sodium acetate buffer, pH 4.5, was applied at a flow rate of 5 μ l/min. Unreacted ester groups were blocked by a 7-min injection of 1.0 M ethanolamine hydrochloride solution (35 μ l). 11,000 RU were obtained with GST, and 6545 RU were obtained for GST-RAGE. For the study of protein-protein interactions, RAGE, sRAGE, and V peptide were diluted in 50 mM Tris buffer (0.15 M NaCl, 5 mM CaCl₂, 0.005% (v/v) P20, pH 7.5) from 0.2 to 12.8 μ M. SPR sample run conditions consisted of a cycle length of 5 min with a flow

rate of 10 μ l/min. At the end of each cycle, the chip surface was regenerated using 50 mM NaOH (10 μ l, 30 s). Measurements of protein interactions were obtained from resulting SPR sensograms and expressed in terms of observed binding to immobilized proteins as measured by arbitrary resonance units (RU).

NF- κ B Luciferase Assay—HEK293T cells (3×10^4 cells per well in 24-well plates) were incubated in 2% FCS-supplemented DMEM for 24 h prior to transfection. Cells were mock-transfected or co-transfected with 2 μ g of cytomegalovirus-driven *Renilla* luciferase plasmid (pRL) and 200 μ g of pGL3 basic vector (Promega) or pNFKB-Luc. 24 h post-transfection, cells were treated with 10 μ g/ml S100B for another 24 h. Cells were lysed in Passive Lysis Buffer (Promega) and subjected to Dual-Luciferase assay (Promega) followed by quantification using a Microplate Plus LB96V luminometer (Berthold). To investigate RAGE blockade by sRAGE and V peptide, cells were incubated with purified GST-tagged sRAGE or V peptide at 24 h post-transfection. 10 μ g/ml S100B was added to the medium containing the recombinant peptides. In parallel, cells were cultured in fresh medium to remove the free peptides in the previous medium before S100B treatment. 24 h after S100B treatment, cells were lysed in Passive Lysis Buffer and subjected to Dual-Luciferase assay.

RESULTS

RAGE Activates MAPK Signaling Pathways in HEK293T Cells—Immunofluorescence and Western blotting analysis demonstrated that RAGE is expressed in HEK293T cells, which

is consistent with the finding that HEK293T cells react with S100 proteins (33). RAGE was localized to the plasma membrane of the HEK293T cells (Fig. 1A), and the RAGE antibody recognized four major bands with molecular masses of 55, 50, and 45 kDa and a RAGE splicing form of 35 kDa, respectively (Fig. 1B). The specificity of the RAGE immunoreactivity was demonstrated by the absence or significant diminution of bands following transfection of HEK293T cells with RAGE-specific siRNA (Fig. 1B).

In comparison with untreated cells, those treated with increasing concentrations of AGE-BSA or S100B demonstrated a RAGE ligand-induced phosphorylation of various protein kinases, including p44/42 (ERK1/2), MEK1/2, p38, JNK, and p90RSK, in a dose-dependent manner (Fig. 1C). In contrast, the phosphorylation level of Akt remained unaltered by RAGE ligand exposure (Fig. 1C). The increased phosphorylation of p44/42 and MEK1/2 in S100B or AGE-BSA-treated HEK293T cells was observed at 10 min and peaked at 1 h post-treatment when compared with the basal phosphorylation levels of p44/42 and MEK1/2 in nontreated cells (Fig. 1D). Control (non-AGE-modified) BSA produced no response (data not shown).

HEK293T cells were transfected with RAGE siRNA to determine whether S100B or AGE-BSA-induced activations of MAPK pathways are RAGE-dependent. RAGE siRNA-transfected HEK293T cells failed to show S100B and AGE-BSA-induced phosphorylation of p44/42, whereas the cells expressing the scrambled siRNA still showed the increased phosphorylation of p44/42 (Fig. 1E).

RAGE V Domain Mediates the Receptor Homo-interaction in Cells or in Vitro—Fluorescence resonance energy transfer analysis of RAGE ligands has indicated that RAGE forms oligomers (23). To further study this phenomenon, the RAGE cDNA was subcloned into pcDNA3 vectors containing GFP or HA tag. GFP-RAGE plasmid was co-transfected with pcDNA3-HA vector or HA-RAGE plasmid. At 24 h post-transfection, cells were treated with S100B or AGE-BSA, and co-immunoprecipitation was carried out with anti-HA antibody and the whole cell lysates. GFP-RAGE was detected by both anti-GFP antibody (green bands) and anti-RAGE antibody (red bands) in GFP-RAGE and HA-RAGE co-transfected cells but was absent in GFP-RAGE and pcDNA3-HA vector co-transfected cells (Fig. 2A). S100B or AGE-BSA treatment further potentiated the binding of GFP-RAGE to HA-RAGE (Fig. 2A). Quantification of the blots indicated that the interaction between RAGE proteins was enhanced by nearly 50% in the presence of 10 μ g/ml S100B or 200 μ g/ml AGE-BSA (Fig. 2B). To further examine the RAGE homo-interaction, RAGE was expressed as a GST fusion protein in *E. coli*, purified, immobilized on glutathione-Sepharose 4B beads, and incubated with the lysates from GFP-RAGE-overexpressing HEK293T cells. GST-RAGE was shown as double bands on SDS-polyacrylamide gel, which is a common phenomenon for prokaryotically expressed GST fusion proteins, including GST-fused RAGE protein (17), and is presumably due to proteolysis of the expressed protein (34). GST pull-down assay indicated that GFP-RAGE interacted with GST-RAGE specifically but not GST control protein *in vitro* (Fig. 2C). Similarly to the co-immunoprecipitation assay, the interaction between GFP-RAGE and GST-RAGE was enhanced in the presence of S100B or AGE-BSA (Fig. 2, C and D).

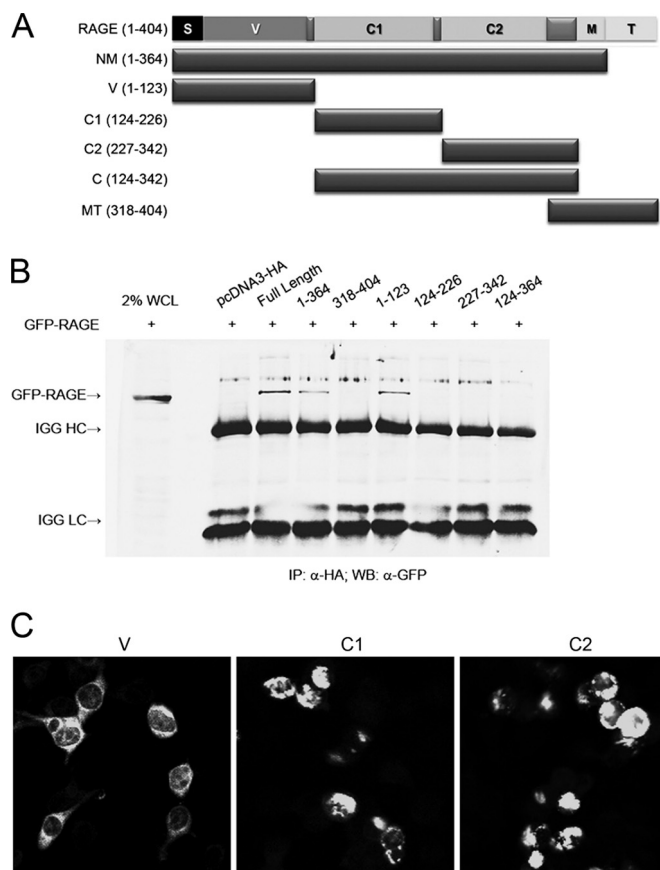


FIGURE 3. RAGE V domain is essential for RAGE homo-interaction. A, schematic representation of RAGE deletions. S, signal peptide; V, IgV-like domain; C, IgC-like domain; M, transmembrane region; T, cytosolic tail. B, GFP-RAGE plasmid was co-transfected with HA-tagged RAGE deletions into HEK293T cells. The co-immunoprecipitation was performed using anti-HA antibody. The immunoprecipitates (IP) were probed with anti-GFP antibody. WCL, whole cell lysate. C, HEK293T cells were grown on coverslips and transfected with GFP-tagged RAGE V, C1, or C2 expression plasmid. 24 h post-transfection, cells were fixed and examined under a fluorescent microscope.

To map the key region that mediates RAGE homo-interaction, plasmids encoding different RAGE deletions were constructed with HA tag (Fig. 3A) and co-transfected with GFP-RAGE in HEK293T cells. Co-immunoprecipitation revealed that the deletions lacking the RAGE N-terminal V domain were incapable of binding to GFP-RAGE (Fig. 3B), indicating that the V domain is indispensable for RAGE homo-interaction. Further evidence for this was obtained from fluorescent microscopy of HEK293T cells that were transfected with GFP-tagged V, C1, or C2 expression plasmid. It was shown that only the RAGE deletion containing V domain was localized to the plasma membrane, indicating the association of this peptide to the cell surface RAGE, although by contrast RAGE C1 and C2 were expressed in the cell cytoplasm (Fig. 3C).

Endogenous RAGE Forms Homodimers at the Plasma Membrane—To identify RAGE oligomers, HEK293T cells were collected and treated with BS³, a water-soluble and membrane-impermeable cross-linker that is ideal for cross-linking of the cell surface proteins. The whole cell lysates were then extracted and subjected to Western blotting analysis. Monomeric RAGE was detected at ~55 kDa, although RAGE protein was also

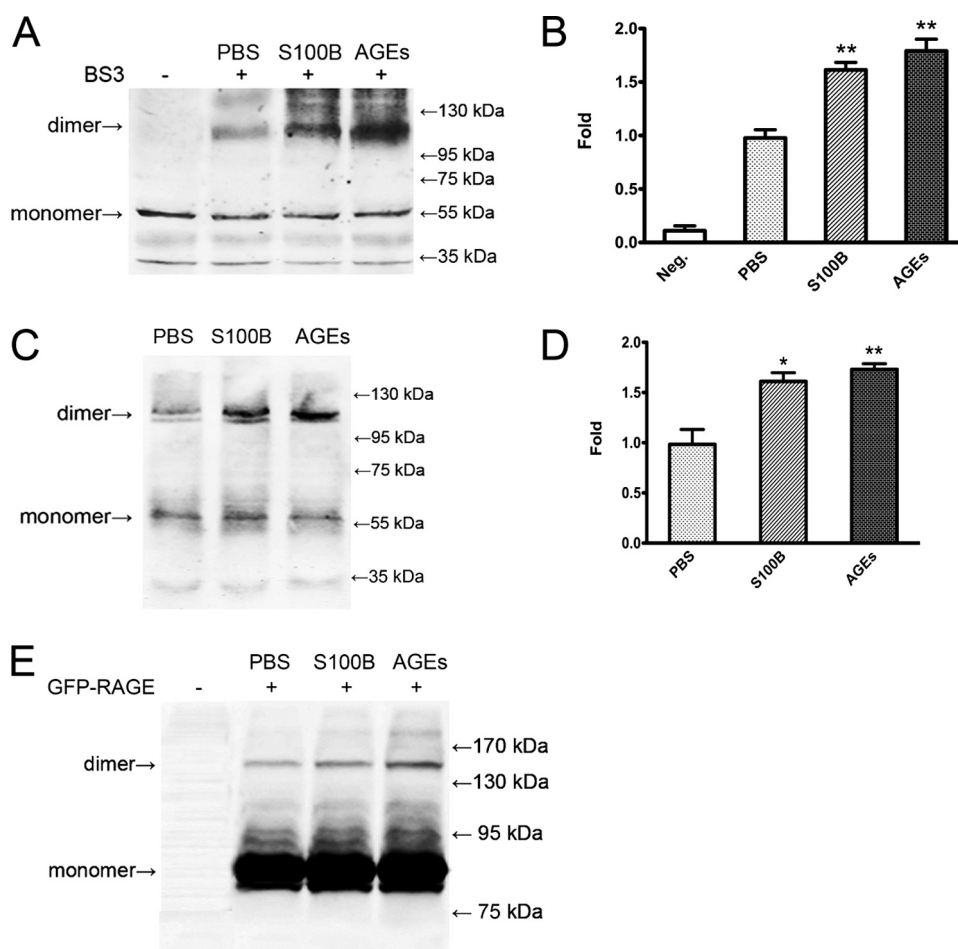


FIGURE 4. Endogenous RAGE forms homodimers at the plasma membrane. A, HEK293T cells were treated with PBS or 10 μ g/ml S100B or 200 μ g/ml AGE-BSA for 30 min. About 2×10^7 /ml of the cells were cross-linked with 4 mM BS³ for 30 min at room temperature. The cross-linking was terminated by adding 20 mM of Tris-HCl. 20 μ g of the lysates from BS³-treated cells was probed with anti-RAGE antibody. B, quantitation of RAGE dimerization as shown in A using Odyssey imaging system. **, $p < 0.01$ for S100B or AGEs-treated cells versus PBS-treated cells. Data are presented as mean \pm S.E. of triplicate experiments. Neg., negative. C, HEK293T cells were treated with PBS or 10 μ g/ml of S100B or 200 μ g/ml AGE-BSA for 30 min. The whole cell lysates were applied to nondenaturing PAGE and probed with anti-RAGE antibody. D, quantitation of RAGE dimerization as shown in C using Odyssey imaging system. *, $p < 0.05$; **, $p < 0.01$ for S100B or AGEs-treated cells versus PBS-treated cells. Data are presented as mean \pm S.E. of triplicate experiments. E, HEK293T cells were transfected with GFP-RAGE expression plasmid. 24 h post-transfection, cells were cultured in 1% FCS/DMEM for another 24 h. The whole cell lysates were subjected to nondenaturing PAGE and Western blotting using anti-GFP antibody.

observed at ~ 110 kDa with some minor bands with higher molecular masses. This suggests that RAGE exists as both monomers and oligomers, although dimers appear to be the dominant oligomeric form of this receptor (Fig. 4A). Consistent with the protein interaction assays, treatment with 10 μ g/ml S100B or 200 μ g/ml AGE-BSA prior to protein cross-linking resulted in $\sim 50\%$ enhancement of RAGE dimerization (Fig. 4B). As a parallel approach to investigate formation of RAGE dimers, a band at ~ 110 kDa was detected in nondenaturing PAGE, and ligand treatment increased the band intensity (Fig. 4, C and D). The lysates from GFP-RAGE-transfected cells were also subjected to the nondenaturing PAGE. The GFP-RAGE monomers at 80 kDa and homodimers at 160 kDa were detected (Fig. 4E). Consistent with the previous results, S100B and AGE-BSA treatments increased GFP-RAGE dimerization compared with the control (Fig. 4E).

control. No binding was observed (RU < 10 , data not shown). These results confirmed that RAGE is capable of binding to another RAGE molecule, its soluble form, or the V peptide, which harbors the key region mediating RAGE homo-interaction.

Soluble RAGE and the Peptide of RAGE V Domain Block RAGE Dimerization and Signaling—Because it has been reported that both sRAGE and blockade of RAGE V domain by the site-specific antibody can inhibit RAGE function (35), we investigated if sRAGE or RAGE V peptide inhibits dimerization of the protein. HEK293T cells were incubated with GST-tagged sRAGE or RAGE V peptide at 37 $^{\circ}$ C for 1 h before cell lysis. The native gel indicated that RAGE dimerization was inhibited in sRAGE or RAGE V peptide-treated cells compared with GST control protein-treated cells (Fig. 6A). Both sRAGE and RAGE V peptide inhibited S100B/RAGE-induced phosphorylation of

Soluble RAGE and RAGE V Peptide Bind to RAGE—Because RAGE homodimerization is mediated by the RAGE V domain, we asked whether sRAGE, which contains the extracellular part of RAGE, could bind to RAGE. The interaction of sRAGE with full-length RAGE was assayed by GST pulldown. GST-fused RAGE, sRAGE, and V peptide, as well as GST control protein, were expressed in *E. coli*, purified, immobilized on glutathione-Sepharose 4B beads, and incubated with the cell lysates from GFP-RAGE-over-expressing cells. As a result, GFP-RAGE was pulled down by GST-tagged RAGE, sRAGE, and V but not GST control protein (Fig. 5A).

SPR experiments were carried out to further analyze the binding between RAGE molecules. GST-RAGE was immobilized onto a CM5 Biacore sensor chip. GST-tagged RAGE, sRAGE, and V peptide were cleaved by thrombin to remove the GST tag (Fig. 5B). These peptides were then injected over the flow cells. The sensorgrams of the three peptides showed a rapid increase of RU indicating binding of these peptides to the immobilized RAGE on the chip followed by a decrease of RU resulting from a loss of the bound molecules upon washing (Fig. 5C). Binding of the immobilized RAGE to RAGE, sRAGE, and V peptide were concentration-dependent (Fig. 5, C and D). RAGE, sRAGE, and V peptide were also injected over the flow cell on which GST was immobilized as a negative

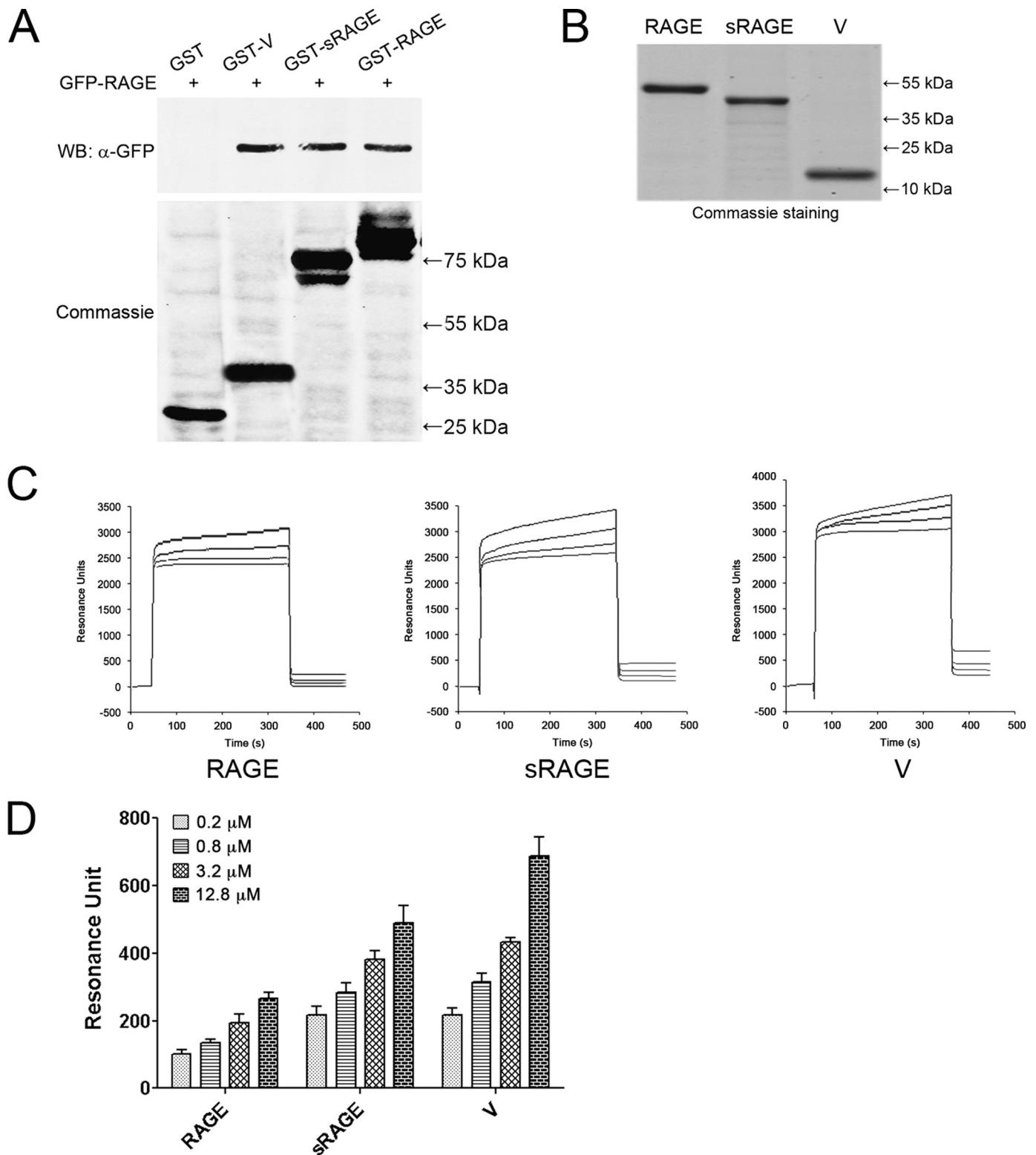


FIGURE 5. Soluble RAGE and RAGE V peptide bind to RAGE. *A*, GFP-RAGE overexpressing cell lysates were incubated with purified and immobilized GST, GST-RAGE, GST-sRAGE, or GST-V peptide. The proteins bound to glutathione-Sepharose 4B beads were extensively washed and eluted by boiling in SDS protein sample buffer. The eluates were applied to the Western blotting (WB) for GFP-RAGE using anti-GFP antibody (*upper panel*) and the immobilized GST fusion proteins were stained with Coomassie Brilliant Blue R250 (*lower panel*). *B*, RAGE, sRAGE, and V peptide were purified from *E. coli* as GST fusion proteins, followed by cleavage with thrombin to remove GST tag. Cleaved proteins (5 μ g) were loaded on a 15% SDS-polyacrylamide gel and staining with Coomassie Brilliant Blue R-250. *C*, GST-RAGE (6545 RU) was directly immobilized onto the CM5 sensor chip. RAGE, sRAGE, or RAGE V peptide (0.2–12.8 μ M) was injected over the flow cell at a flow rate of 10 μ l/min for 5 min for analysis of sensorgrams. *D*, binding abilities (RU) of RAGE, sRAGE, and V to immobilized GST-RAGE assayed by SPR are shown as a bar chart. Data are means \pm S.E. of triplicate injections.

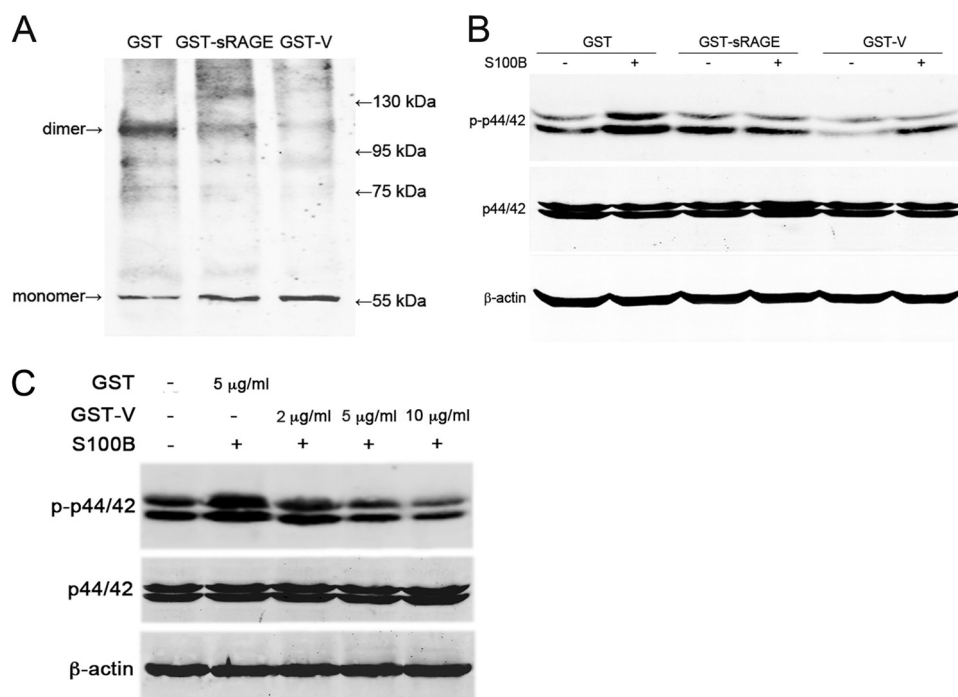


FIGURE 6. Soluble RAGE and RAGE V peptide block RAGE dimerization and subsequent MAPK activation. A, HEK293T cells were incubated with 5 μ g/ml GST, GST-sRAGE, or GST-V peptide for 1 h prior to treatment with 10 μ g/ml S100B for 30 min. The whole cell lysates were subjected to nondenaturing PAGE, followed by Western blotting for RAGE. B, HEK293T cells were incubated with 5 μ g/ml GST-sRAGE or GST-V peptide for 1 h prior to treatment with 10 μ g/ml S100B for 30 min. The cell lysates were subjected to Western blotting for β -actin, total, and phosphorylated p44/42. C, HEK293T cells were incubated with 5 μ g/ml GST or the indicated amount of GST-V peptide (2–10 μ g/ml) for 1 h prior to treatment with 10 μ g/ml S100B for 30 min. Total and phosphorylated p44/42 as well as β -actin were detected by Western blotting using 20 μ g of the whole cell lysates.

p44/42 MAPK (Fig. 6B), and a dose-dependent inhibition of S100B/RAGE-induced p44/42 phosphorylation by RAGE V peptide was observed (Fig. 6C).

Blockade of RAGE by Soluble RAGE or RAGE V Peptide Attenuates NF- κ B Activation—It has been well documented that ligand binding to RAGE leads to NF- κ B transcriptional activation (19), a response that can be attenuated by the extracellular domain of RAGE (ex-RAGE) (21). The inhibition of MAPK pathway by sRAGE and V peptide suggested that the NF- κ B pathway, which is responsible for the expression of some proinflammatory cytokines in response to RAGE activation, might also be blocked. The NF- κ B reporter plasmid pNFkB-Luc or pGL3 basic vector was co-transfected with pRL, the control *Renilla* luciferase plasmid, into HEK293T cells to evaluate the basal activity of NF- κ B in HEK293T cells. At 24 h post-transfection, cells were treated with S100B for another 24 h. The Dual-Luciferase assay showed a relatively high NF- κ B transcriptional activity in pNFkB-Luc-transfected HEK293T cells compared with pGL3 basic vector-transfected cells (>5,000-fold) ($p < 0.001$), which was increased by 2.1-fold following S100B exposure ($p < 0.01$) (Fig. 7A). To investigate the inhibitory effects of sRAGE and V peptide on NF- κ B, HEK293T cells carrying NF- κ B reporter plasmid were incubated with purified GST-tagged sRAGE, V peptide, or GST control protein for 1 h prior to the addition of S100B into the medium. Alternatively, cell culture medium was replaced with fresh medium to remove free peptides before S100B treatment to prevent competitive ligand binding. Compared with mock-treated cells,

GST did not affect the basal and S100B/RAGE-induced NF- κ B activity. In the presence of sRAGE or V peptide, the basal activity of NF- κ B pathway was not obviously altered, but S100B/RAGE-induced NF- κ B activation was significantly inhibited by 44 or 37%, respectively, in contrast to GST-treated cells ($p < 0.01$). In the case that free sRAGE or V peptide was removed from the culture medium after a 1-h preincubation, S100B/RAGE-induced NF- κ B activation was still decreased by 29% ($p < 0.01$) or 24% ($p < 0.05$), respectively, compared with GST-treated cells (Fig. 7B). In combination with the previous data, these results indicate that S100B/RAGE-induced NF- κ B activation can be prevented by sRAGE and V peptide not only through competitive ligand binding but also through other mechanisms, e.g. blockade of intrinsic RAGE homodimerization.

DISCUSSION

RAGE is widely expressed as a cell surface glycoprotein in many cells and tissues. Although the precise nature of receptor-ligand interactions and downstream signaling is still being elucidated, it is well known to modulate important proinflammatory responses (36, 37). Although the calculated molecular mass of RAGE is ~42 kDa, the actual bands detected by SDS-PAGE and Western blotting are ~45, 50, and 55 kDa. The specificity of the bands detected by the RAGE antibody was confirmed by combining an siRNA approach to knock down RAGE in cells. The major band at 55 kDa may be due to the post-translational modifications such as *N*-linked glycosylation (38, 39), a common feature of cell surface receptors. HEK293 cells were used as an *in vitro* model because it has been previously demonstrated that they react to S100 proteins (33). We have also now demonstrated that they express constitutive levels of RAGE. Exposure of HEK293T cells to the RAGE ligands S100B and AGE-BSA induced the phosphorylation of MEK1/2, p44/42, JNK, p38, and p90RSK, which is in common with many reported studies demonstrating RAGE-initiated signaling, although the nature of these transduction cascades evoked may differ between distinct cell types (24).

It is well established that S100B exists in oligomeric form, including dimers and tetramers (26). Indeed, S100B tetramers bind RAGE with higher affinity than dimeric S100B, and such oligomers may be able to induce dimerization of RAGE at the V domain (26). Furthermore, a putative RAGE tetramer is induced by the interaction with S100A12 hexamers (30). In this study, a combination of co-immunoprecipitation, GST pull-down, and SPR has demonstrated the interaction between RAGE monomers via the V domain, which was further poten-

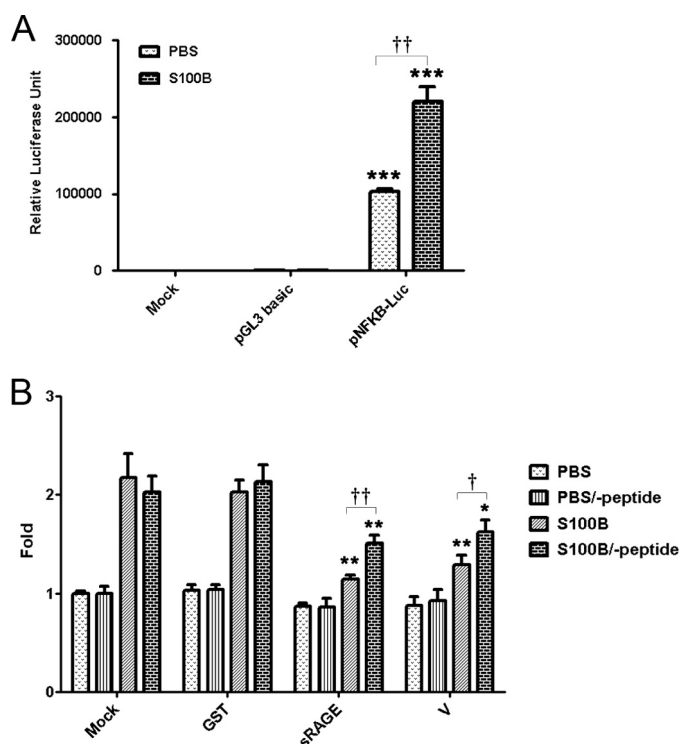


FIGURE 7. Soluble RAGE and RAGE V peptide inhibit S100B/RAGE-induced NF- κ B pathway. A, HEK293T cells were maintained in 2% FCS containing DMEM and mock-transfected or transfected with 2 μ g of pRL and 200 μ g of pGL3 basic vector or pNF κ B-Luc. 24 h post-transfection, cells were treated with PBS or 10 μ g/ml S100B. Cells were lysed in passive lysis buffer, and luciferase activities were measured after 24 h of treatment. ***, $p < 0.001$ for pNF κ B-Luc-transfected versus pGL3 basic vector-transfected cells. ††, $p < 0.01$ for S100B versus PBS-treated cells. B, HEK293T cells were grown in 2% FCS supplemented DMEM and transfected with 2 μ g of pRL and 200 μ g of pNF κ B-Luc. 24 h post-transfection, cells were mock-treated or preincubated with 5 μ g of GST, sRAGE, or V peptide for 1 h followed by treatment with PBS or 10 μ g/ml S100B for 24 h (shown as S100B/+peptide). Alternatively, at 1 h after preincubation with GST, sRAGE, or V peptide, the culture medium was replaced with fresh medium before S100B treatment (shown as S100B/-peptide). *, $p < 0.05$; **, $p < 0.01$ for sRAGE- or V-preincubated cells versus GST-preincubated cells. †, $p < 0.05$; ††, $p < 0.01$ for S100B treatment in sRAGE- or V peptide-containing medium versus fresh medium. Data are means \pm S.E. of triplicate cultures and are representative of three independent experiments.

tiated by ligand binding. In nondenaturing gels and SDS-PAGE loaded with cross-linked proteins, the predominant high molecular mass receptor species is ~ 110 kDa, which represents a dimeric fusion of RAGE monomers. Such dimerization appears to occur constitutively, but upon exposure to RAGE ligands, it is appreciably enhanced. It should be noted that monomeric RAGE can also be detected in native gels and SDS-PAGE separating extracts of plasma membrane cross-linked proteins. This suggests that RAGE may not function exclusively in its dimeric form. In addition, some minor >110 -kDa bands were observed in cross-linked protein samples indicating that RAGE might form oligomers or possibly even heterodimers with other cell surface proteins, although precisely how this regulates intracellular signaling remains uncertain.

Our study indicates that dimerization of RAGE is an important step in receptor signaling following ligand binding, as shown by MAPK phosphorylation and downstream transcriptional activation. The mechanism for this remains to be identified, but it seems likely that dimerization offers the receptor a better recognition of its ligands, especially if they also occur in

oligomeric configurations. Although RAGE C-terminal region is not homologous to any known kinase domains, receptor dimerization and its potentiation by ligand binding might have an effect on the association of RAGE cytosolic tail to its interacting proteins, e.g. Dia-1, to trigger signal transduction.

Various methods to block RAGE signaling have been explored. Beyond the reported benefits of sRAGE acting as a competitive inhibitor of receptor-ligand binding (14), proinflammatory pathology can be also inhibited by direct receptor blockade with a RAGE V domain-specific antibody (40). Efficacy of this antibody-based neutralization is through blockade of the RAGE V domain and has been shown to attenuate amyloid- β -induced neuronal apoptosis (35).

Receptor homodimerization is important for signal transduction of many receptor classes. For example, G protein-coupled receptors not only form homodimers but also bind to other proteins, including receptors, ion channel, and chaperones (41). Interference with this dimerization has been central to the development of therapeutic small molecule inhibitors (42). The current investigation has further explored the nature of RAGE dimerization in response to ligand binding and identified the protein domains responsible. We have demonstrated that in HEK293T cells, RAGE binding to S100B and AGEs leads to enhanced dimer formation and that this is intimately linked to signal transduction and transcriptional activation. RAGE signaling and NF- κ B activation can be effectively attenuated by preventing receptor dimerization using sRAGE and a RAGE V peptide, both of which showed concentration-dependent binding to RAGE as determined by SPR. Removal of free sRAGE and V peptide from the cell culture medium after preincubation still revealed a decrease in NF- κ B transcriptional activity. This indicates that sRAGE and V peptide might not only competitively bind to RAGE ligand to exert their inhibitory effects but also act in a more direct way to the endogenous RAGE, e.g. binding to cell surface RAGE to block homodimerization. It cannot be entirely excluded that some V peptide may be released from heterodimers of recombinant V peptide and endogenous RAGE to block ligand binding. Nevertheless, the V domain has been identified as central to RAGE dimerization. The use of sRAGE or V peptide prevents normal function, not only by acting as a decoy but also by direct blockade of the receptor homo-interaction.

Taken together, our data suggest that RAGE functions as homodimers on the plasma membrane, a phenomenon that may contribute to ligand recognition and signal transduction. Blockade of this mechanism could be an important strategy for the regulation of RAGE-mediated proinflammatory signaling in various disease states.

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Homodimerization Is Essential for the Receptor for Advanced Glycation End Products (RAGE)-mediated Signal Transduction

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J. Biol. Chem. 2010, 285:23137-23146.

doi: 10.1074/jbc.M110.133827 originally published online May 26, 2010

Access the most updated version of this article at doi: [10.1074/jbc.M110.133827](https://doi.org/10.1074/jbc.M110.133827)

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